

GalSafe® Pigs

Environmental Assessment

In support of an approval of a New Animal Drug Application (NADA) for the *pPL657* rDNA construct in the glycoprotein *galactosyltransferase alpha 1,3* gene (*GGTA1*) in the hemizygous and homozygous GalSafe® lineage of domestic pigs (*Sus scrofa domesticus*) resulting in undetectable endogenous galactose- α 1,3-galactose sugar residues on biological derivatives of the homozygous GalSafe® pigs that are intended to be used as sources of food or human therapeutics including excipients, devices, drugs or biological products

Prepared by



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List of Acronymns

AAALAC - Association for Assessment and Accreditation of Laboratory Animal Care International.....	19
alpha-gal - galactose- α 1,3-galactose	8
APHIS - Animal and Plant Health Inspection Service	19
AVMA - American Veterinary Medical Association	26
CFR - Code of Federal Regulations	12
CVM - Center for Veterinary Medicine	12
EA - environmental assessment.....	11
EIS - environmental impact statement	12
EPA - Environmental Protection Agency.....	31
FD&C Act - Federal Food, Drug, and Cosmetic Act.....	12
FDA - Food and Drug Administration.....	7
FONSI - finding of no significant impact	12
G0 – ear tag label that correspond to the absense of pPL657 or the nonengineered genotypic identity	25
G1 – ear tag label that correspond to pPL657 on one allele of GGTA1 or the hemizygous GalSafe® genotypic identity.....	25
G2 – ear tag label that correspond to pPL657 on both alleles of GGTA1 or the homozygous GalSafe® genotypic identity.....	25
GGTA1 - glycoprotein α 1,3-galactosyltransferase gene	8
GRAS - Generally recognized as safe	43
GT - galactosyltransferase.....	11
ID – identification.....	25
IDNR - Iowa Department of Natural Resources.....	28
IgA - immunoglobulin isotype A.....	8
IGA - intentional genomic alteration	7

IgE - immunoglobulin Isotype E	9
IgG - immunoglobulin isotype G	8
IgM - immunoglobulin isotype M	8
IRES neo poly A - Internal Ribosome Entry Site-Neomycin-bovine growth hormone polyadenylation	14
LR-PCR - Long Range Polymerase Chain Reaction	15
MPP – Meat and Poultry Products	31
NADA - New Animal Drug Application	8
NEPA - National Environmental Policy Act	7
NPDWS - National Primary Drinking Water Standards.....	20
NPTII - aminoglycoside 3'-phosphotransferase II protein; expression product of the nptII gene	7
<i>nptII</i> - neomycin phosphotransferase II gene	7
NRC - National Research Council	37
SCNT - somatic cell nuclear transfer	15
SCWDS - Southeastern Cooperative Wildlife Disease Study	34
SMG - selectable marker gene.....	15
SOP - standard operating procedure	19, 20
USC - United States Code	12
USDA - United States Department of Agriculture	19

Section 1.0 Overview

This document constitutes an assessment of potential environmental impacts posed by an intentional genomic alteration (IGA) in a line of pigs, referred to as GalSafe® pigs, which include both homozygous and hemizygous pigs. Homozygous GalSafe® pigs are intended to be used as sources of food or human therapeutics including excipients, devices, drugs or biological products. The IGA is the *pPL657* rDNA construct in the glycoprotein *galactosyltransferase alpha 1,3* gene (*GGTA1*), which results in undetectable endogenous galactose- α 1,3-galactose (alpha-gal) sugar residues on biological tissues in these swine. The EA addresses requirements stipulated under the National Environmental Policy Act (NEPA) and in FDA regulations (21 CFR Part 25 and 21 CFR 514.1(b)(14)) to determine whether the agency action (i.e., approval of the article meeting the definition of a new animal drug) is likely to have a significant impact on the human environment of the United States.

GalSafe® pigs are bred, farrowed, and finished at a single production facility located in northern Iowa; following humane euthanasia of the homozygous GalSafe® pigs, selected tissues (manufacturing intermediates) are removed, packaged, labeled, and shipped to Revivacor's business partner for subsequent fabrication and packaging as human therapeutics. Alternatively, homozygous GalSafe® pigs may be transported to a single United States Department of Agriculture (USDA) inspected abattoir (slaughterhouse) located in southern South Dakota for subsequent processing into food, as well as the collection of manufacturing intermediates.

As described herein, two general exposure pathways that could result in environmental impacts were identified and evaluated in this assessment:

1. The escape* of GalSafe® pigs from the single production facility in Iowa and single abattoir (slaughterhouse) in South Dakota into the affected environment, and
2. The introduction of the neomycin *phosphotransferase II* gene (referred to herein as the *nptII* gene) that confers neomycin resistance and its expression product, aminoglycoside 3'-phosphotransferase II (referred to herein as the NPTII protein), resulting from the IGA, into the natural environment via manure or other wastes (including carcasses and any remnants) from the GalSafe® pigs at the single production facility in Iowa and single abattoir in South Dakota.

* Herein, escape includes the potential for unintentional release; e.g., by malicious activities or natural disaster.

These exposure pathways and their potential associated risks are discussed and evaluated in the following sections taking into account a series of risk-related questions that address the likelihood of escape, survival, dispersal, reproduction and establishment of GalSafe® pigs in the affected environment. Consideration is also given to the risk from toxicity and the likelihood of an increase in the presence of antimicrobial resistance occurring due to the possible presence of the *nptII* gene and NPTII protein in manure and other waste products.

Details are provided in this assessment that illustrate the steps that Revivacor has taken to ensure containment of the GalSafe® pigs, and limit exposure to the *nptII* gene and NPTII protein. Based on these considerations, which would be included in the conditions of approval should one be granted, an approval of the NADA for the *pPL657* rDNA construct in the genome of the GalSafe® pig line is unlikely to result in a significant impact on the human environment of the United States.

1.1 Background

Most mammalian species (including New World monkeys, cows, pigs, and mice) express galactose- α 1,3-galactose (alpha-gal) on cells and tissue surfaces.¹⁻⁴ Alpha-gal expression results from the activity of an enzyme encoded by the glycoprotein *α 1,3-galactosyltransferase* gene (*GGTA1*).^{1-3, 5} Certain mammalian species, such as catarrhines (humans, apes, and Old World monkeys), do not have a functional *GGTA1* gene⁶⁻⁸ and correspondingly do not express alpha-gal.^{1, 3, 4} Additionally, alpha-gal has been documented to be absent in fish, amphibians, reptiles, and birds.^{3, 9, 10} The function of alpha-gal is unknown³, but is clearly not essential for survival in the modern world.^{1, 3}

Immunoglobulins (Ig), otherwise known as antibodies, play a role in the body's immune system. They attach to foreign substances such as bacteria, and assist in destroying them. Typically, they are segregated into classes or types including IgA, IgG, IgM, IgD, and IgE. Mammalian species that do not produce alpha-gal, such as humans and old world primates, have been well documented to possess natural anti-alpha-gal antibodies.^{1-3, 11, 12} The occurrence of anti-alpha-gal IgG and IgM antibodies in humans has been attributed to gastrointestinal bacterial flora that express alpha-gal and are constantly challenging the host's immune system.¹² It has been reported that the antibody is present as immunoglobulin isotypes of IgG, IgM, and IgA.^{1, 2, 13} The antibodies have been previously characterized at birth to be absent (IgM)¹⁴, or at reduced quantities (IgG; attributed to placental transfer),^{14, 15} but develop thereafter.^{14, 15} The primary role of these antibodies is to initiate a response to an antigen that is responsible for a pathogenic state (predominantly IgM) and subsequently provide long term immunity to that antigen or antigen with similar conformation (IgG). In humans, anti-alpha-gal antibodies are one

of the most abundant immunoglobulins, with some studies reporting that 1-3% of circulating immunoglobulins are directed to alpha-gal.^{3, 11-15}

IgE antibodies have specificity to a unique antigen and are responsible for allergic response that includes anaphylaxis. Anaphylaxis paired with anaphylactic shock is a life threatening condition that may be fatal; subjects must seek immediate emergency care. Typical symptoms include urticaria, tachycardia, angioedema, syncope, and hypotension. More recently, allergists¹⁶⁻²² have described large populations with high titers of anti-alpha-gal IgE that appears to be initiated by arachnid bites.

Populations are usually categorized as:

- Allergen status is positive (anti-alpha-gal IgE > threshold).
- Allergen status is negative (anti-alpha-gal IgE < threshold).

The reported prevalence of individuals in regions of the the United States with elevated allergen specific titers of anti-alpha-gal IgE (e.g. allergen positive) has been reported to be in the range of 8% to 46% with highest prevalence commensurate with the geographical range of the arachnid responsible for initial sensitization (Figure 1).^{19, 23-26} Males appear to have a higher prevalence than females, but no formal correlation between gender and alpha-gal sensitization has been established.²⁰ Similar observations have been reported for prevalence in other regions around the world.²⁷⁻²⁹ Children within the geographic range of the arachnids are projected to have allergen positive prevalence comparable to the adult population.³⁰ As one might expect, hunters and forest service workers have been reported to have a prevalence that is more than twice the general population.^{19, 29}

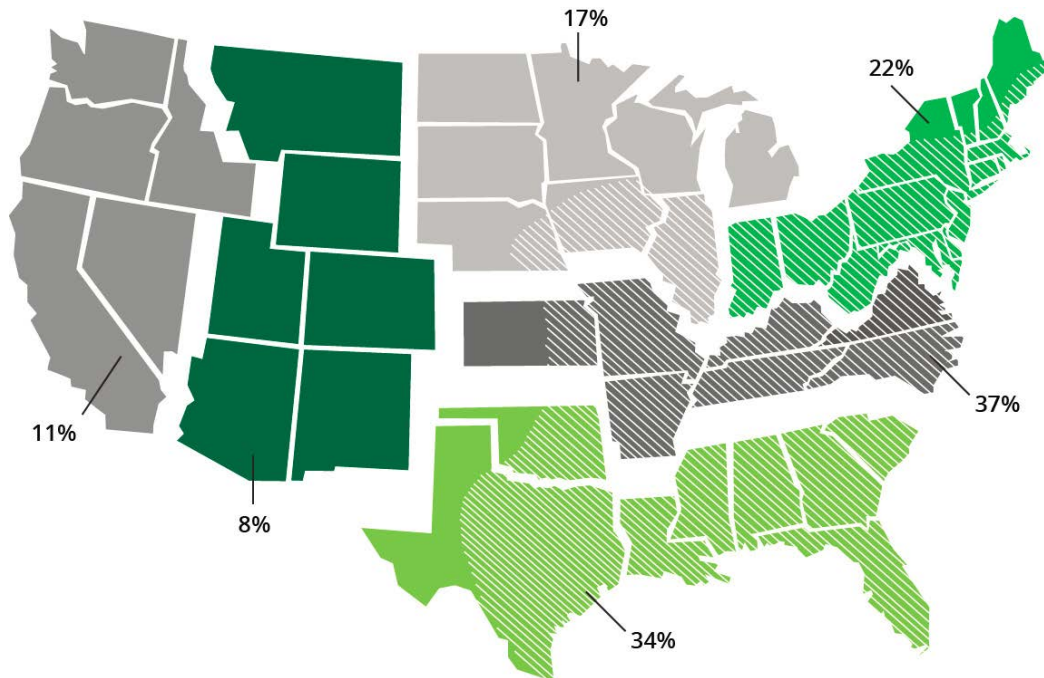


Figure 1. Prevalence of IgE to alpha-gal. Percent positive rates are presented for IgE to alpha-gal within each of six regions in the United States, 2012-2013 (7300 samples). Diagonal white lines on the map represent the known geographic distribution of the Lone Star tick [from Olafson, P. Ticks and the mammalian meat allergy. USDA Beef Research, (2015)].²⁶

The Lone Star tick (*Amblyomma americanum*) is the primary culprit in the United States for anti-alpha-gal IgE sensitization, however, the blacklegged tick (*Ixodes scapularis*) and chiggers³¹ (*Trombiculidae*) have been implicated. Importantly, bites from certain other tick species (*Ixodes ricinus*, *Ixodes holocyclus*, *Amblyomma cajennense*, *Amblyomma sculptum*, and *Haemaphysalis longicornis*) around the world have been documented to initiate a similar hypersensitivity to alpha-gal.^{21, 22, 28, 32-37} Of note, *Haemaphysalis longicornis* or longhorned tick is native to East Asia, however, it was recently identified by the CDC to be present in 9 different states.³⁸ Its range is expected to expand rapidly to a substantially larger area than its current locations.³⁹ Concomitantly, other ticks, including the Lone Star tick, are also reported to expand their range due to climate change and the expanding deer population.⁴⁰ As the range of these ticks expands, more individuals will potentially be sensitized to alpha-gal. Additionally, other vectors of IgE sensitization are likely to be revealed as new discoveries are made.

Enrichment for a non-functional *GGTA1* gene and resulting absence of alpha-gal from catarrhines may have been due to a selective evolutionary event prompted by an infectious agent that occurred approximately 28 million years ago.^{1, 3, 9, 41} It appears to have been isolated to primates that

resided within distinct geographical boundaries that are considered the “Old World.”^{1, 3, 9, 41} The loss of immune tolerance to alpha-gal and, correspondingly, the production of anti-alpha-gal antibodies has been postulated to be an essential immunological defense and ultimately vital for the survival of the species under the duress of the infectious agent present during this time.^{1, 3, 9, 41}

The fact that alpha-gal has been verified on cells and tissues of animals without the IGA has significant clinical implications in discordant[†] transplantation (i.e., pig to human, pig to old world primate).^{1, 2, 4} This is due to the fact that alpha-gal is the major antigen expressed on pig cells and tissues to which natural anti-alpha-gal antibodies bind.^{3, 9, 13, 42} The binding of anti-alpha-gal antibodies to alpha-gal activates the complement system within minutes to hours of discordant tissue, cell, or organ transplantation and the host effectively rejects the transplanted material.^{2, 4} Nonetheless, anti-alpha-gal immunoglobulin titers of IgG and IgM may be attenuated by a vegetarian diet while implantation of mammalian-sourced bioprosthetic heart valves increases titers.^{43, 44} The alpha-gal IgE sensitized individuals have been well documented to experience adverse events including anaphylactic shock^{16-19, 21, 22, 28-30, 32, 45-54} after exposure to alpha-gal via inhalation, consumption, external contact, injection, or implantation of mammalian derived food, cosmetic, or drug products. Anaphylaxis triggered by exposure to alpha-gal was reported to be more prevalent than all other food allergens combined.⁵⁵

Revivacor, Inc. (Blacksburg, VA) has utilized its expertise in somatic cell nuclear transfer (SCNT), in combination with gene targeting techniques to establish a unique proprietary line of pigs containing an IGA resulting in undetectable alpha-gal. Revivacor accomplished this via disruption of the pig *GGTA1* locus mediated by *pPL657* vector that targeted exon 9, the location encoding the catalytic domain of the galactosyltransferase (GT) enzyme.⁵⁶ Homozygous inactivation of both alleles of *GGTA1* results in an inactive GT enzyme, thus alpha-gal is undetectable in these pigs.⁵⁷ This technology and subsequent breeding has resulted in the development of a line of pigs, GalSafe®. The intended use of GalSafe® pigs is as a source of food products for human consumption as well as a source of human therapeutics such as excipients, devices, drugs, or biological products.

1.2 Description of the product

The product of this environmental assessment (EA), and subject for regulatory approval under an NADA, is the *pPL657* rDNA construct in the genome of the GalSafe® lineage of domestic pigs that are

[†] “Discordant transplantation” refers to a transplant between members of very different species.

bred, farrowed, and finished at a single site in northern Iowa under strict conditions of physical containment. Homozygous GalSafe® pigs intended for food use are securely transported to a single USDA inspected abattoir in southern South Dakota for processing into food products under the regulatory authority of the USDA, or collection of manufacturing intermediates. Following euthanasia, selected tissues will be removed, packaged, labeled, and shipped to Revivacor's business partner for subsequent fabrication and packaging as a human therapeutic that will be subject to separate FDA regulatory approvals.

1.3 The proposed action: an NADA approval for the pPL657 rDNA construct in the GalSafe® pig

1.3.1 Regulatory mandate

As described in FDA Guidance for Industry 187⁵⁸, intentionally altered genomic DNA in animals is regulated under the new animal drug provisions of the Federal Food, Drug, and Cosmetic Act (FD&C Act), and CVM has established a risk-based hierarchical approach for demonstration of safety and effectiveness that is consistent with FD&C Act (21 USC 321 et seq.) and its enabling regulations (21 CFR 511 & 514).

This approach begins with a product definition, and proceeds through a step-wise series of investigations to characterize the potential hazards associated with the rDNA construct, the lineage of the animal with the IGA, and the durability of its genotype and phenotype. This information enables CVM to determine the likelihood and potential severity of impacts on animal or human health and the environment. Major agency actions, such as an NADA approval, trigger the requirement for preparation of an EA addressing the potential environmental impacts of that action under the NEPA. This current document constitutes an assessment of potential environmental impacts that: (1) satisfies the Applicant's obligations under the Environmental Impact technical section; (2) addresses NEPA-related responsibilities of the Applicant and FDA described in 21 CFR Part 25; and, (3) provides material assistance to the FDA for making a decision whether to prepare a finding of no significant impact (FONSI) or an environmental impact statement (EIS).

1.3.2 Purpose and need for the proposed action

Revivacor is in the process of requesting an FDA approval (i.e., the proposed action) for the pPL657 rDNA construct in the GalSafe® pig line with the animals intended to be used as a source of food or human therapeutics including excipients, devices, drugs or biological products. In doing so, Revivacor

intends to address a medical need for safer and more efficacious source animals for use as food products, as well as therapeutic products for patients in need.

1.3.3 Approach to assessment

Two general exposure pathways that could result in environmental impacts were identified and their risks were evaluated in this assessment:

1. The escape of GalSafe® pigs from the single production facility in Iowa and single abattoir (slaughterhouse) in South Dakota into the affected environment, and
2. The introduction of the *nptII* gene and the NPTII protein, resulting from the IGA, into the affected environment via manure or other wastes (including carcasses and any remnants) from GalSafe® pigs at the single production facility in Iowa and single abattoir in South Dakota.

The escape of GalSafe® pigs (first exposure pathway) could result in the establishment of a population of pigs with the IGA in the United States. To evaluate this risk, one needs to understand the process of the introduction of the IGA and the production of GalSafe® pigs. Therefore, information on the molecular, phenotypic and genotypic characterization of the product (Section 2.0), as well as the production, containment and waste disposal at the single production facility and single abattoir (Section 3.0) is discussed herein. In addition, the potential environment that could be affected (Section 4.0) and the assessment of risk and impacts (Section 5.0) are also discussed.

The introduction of the *nptII* gene and the NPTII protein into the environment (second exposure pathway) could result in toxicity or an increase in the transmission of antimicrobial resistance in the human environment. To evaluate this risk, information on the presence of the *nptII* gene and the NPTII protein (Section 2.0), waste disposal at the single production facility and single abattoir (Section 3.0), and the potential for antimicrobial resistance to occur in the natural environment (Section 5.0) is discussed.

These two general exposure pathways and their potential associated risks are discussed and evaluated taking into account the following series of risk-related questions:

- What is the likelihood that GalSafe® pigs will escape the conditions of confinement?
- What is the likelihood that GalSafe® pigs will survive and disperse if they escape the conditions of confinement?
- What is the likelihood that GalSafe® pigs will reproduce and establish if they escape the conditions of confinement?

- What are the likely consequences to, or effects on, the environment should GalSafe® pigs escape the conditions of confinement?

In addition,

- What is the risk from toxicity due to the presence of the *nptII* gene and NPTII protein to the affected environment?
- What is the likelihood of increased antimicrobial resistance in the affected environment occurring due to the possible presence of the *nptII* gene and the NPTII protein in manure and other waste products of GalSafe® pigs?

Overall, the risk of potential impacts were found to be minimal due to redundant containment measures (physical, procedural, and biological), as well as limited exposure due to a limited number of GalSafe® pigs (up to 1,000 animals) contained at only one facility and one abattoir (Section 6.0).

Section 2.0 The Product

2.1 Product definition

The product is defined as “*pPL657* in the glycoprotein *galactosyltransferase alpha 1,3* gene (*GGTA1*) in the hemizygous and homozygous GalSafe® line of *Sus scrofa* resulting in undetectable endogenous galactose- α 1,3-galactose sugar residues on biological derivatives of the homozygous GalSafe® line that are intended to be used as sources of food or human therapeutics including excipients, devices, drugs, or biological products.”

2.2 Molecular characterization of the rDNA construct

A *GGTA1* fragment derived from porcine (standard domestic pig) genomic DNA in fetal fibroblasts was utilized to construct the knockout vector *pPL657* in several steps. The *GGTA1* fragment was inserted into a commercially available plasmid using standard cloning techniques. Subsequently, the vector was further genetically engineered to allow for linearization before transfection and to insert an Internal Ribosome Entry Site-Neomycin-bovine growth hormone polyadenylation (IRES neo poly A) fragment. The knockout vector *pPL657*, including the plasmid backbone, does not contain any mobilizable sequences from pathogens including viruses, or substances likely to dysregulate the growth of cells, tissues or organs that are endemic to swine, humans or any other species.

The *nptII* gene is used as a selectable marker gene (SMG)[‡] and is present in the construct that remains in the GalSafe® pigs. This gene is commonly used in molecular biology, particularly in molecular cloning, in which transformed bacteria used to produce the desired construct can be selected from among non-transformants. The *nptII* gene was originally described as part of a transposable element in *E. coli*, transposon Tn5.⁵⁹⁻⁶¹ It allows resistance via the metabolization of certain antibiotics of the aminoglycoside class such as kanamycin, neomycin, or geneticin.^{59, 62, 63} It has been successfully integrated into the genome of organisms as diverse as bacteria, yeasts, plants^{59, 63-67}, and animals^{62, 68} including pigs⁵⁷ and cows.^{69, 70} Additionally, the *nptII* gene has been used as the SMG for gene therapy in human clinical trials.⁷¹

The NPTII protein is an enzyme expressed by the *nptII* gene and confers resistance to certain aminoglycoside antibiotics.⁷² It catalyzes the addition of a phosphate from adenosine triphosphate (ATP) to the 3'-hydroxyl group of an aminoglycoside. This addition causes bacterial resistance to aminoglycoside antibiotics by reducing the antibiotic's affinity for the bacterial ribosome. In other words, if the aminoglycoside does not bind to the ribosome due to the addition of a phosphate, bacterial protein synthesis continues and the bacteria will continue to replicate. The risks of the *nptII* gene and the NPTII protein to the human environment are discussed below in Section 5.3

2.3 Characterization of the targeted insert in the GalSafe® lineage

The IGA, targeted insertion at one allele of *GGTA1*, was achieved *ex vivo* and ultimately *in vivo*. Cell lines from standard domestic pigs (containing no IGA) were transfected with the knockout vector *pPL657* in order to establish cell lines containing the IGA, such that *GGTA1* has been functionally inactivated via the targeted insertion. Subsequently, three of these cell lines were selected as the source of donor cells for SCNT and resulted in several litters of founder pigs. Five of the founder pigs, the lineage progenitors, were selected to establish a herd of pigs containing the IGA through typical breeding practices.

An evaluation of the genomes of the lineage progenitors was performed to ensure the targeting vector integrated only at the targeted site. Molecular characterization of the genome of the lineage progenitors verified (via LR-PCR) and confirmed (via Southern blot) the intended insertion occurred at the intended site. Concomitantly, the genomes of the lineage progenitors were probed in order to

[‡] A selectable marker gene is inserted into a vector and its expression allows for the identification of cells that have been transformed or transfected.

verify that random integration of knockout vector *pPL657* did not occur. Additionally, to further rule out off-target vector integration, the genomes were probed to demonstrate the absence of the plasmid backbone, as well as fragments of the backbone.

The targeted insertion is stably and conservatively transmitted through multiple generations. Genotyping results from 1586 piglets representing 208 litters from lineage progenitors to the F11 generation confirmed that the targeted insertion is stably transmitted to progeny through normal breeding and conforms to Mendelian inheritance. Additionally, an evaluation of the sequence of the targeted insertion demonstrated the targeted insertion was stably transmitted from the knockout vector *pPL657* to cell lines and to a founder pig. Stability of the targeted insertion was substantiated further by confirmation that the sequence of the targeted insertion from a late generation (F4) GalSafe® pig was substantially equivalent to the sequence in a founder pig as well as substantially equivalent to the sequence in the targeting vector.

The results confirmed the lack of secondary, non-targeted random gene or gene fragment insertion events in the lineage progenitor animals, and thus, abrogate the risk of any potential off-target adverse effects associated with knockout vector *pPL657*. Additionally, the data confirm that the targeted insertion is stably transmitted to progeny via normal Mendelian inheritance.

2.4 Phenotypic characterization

The phenotype of the GalSafe® pig is similar to that generally observed in comparators without the IGA, with the exception of the intended knockout of endogenous galactose residues. With regard to the phenotypic characterization, the presence of the targeted insertion in GalSafe® pigs has no detectable difference on the physiology of GalSafe® pigs compared with comparators without the IGA. Changes in gene expression (that could result in toxicity due to aberrant protein expression), changes in immune function (such as causing susceptibility to pathogens), and changes in metabolic “setpoints” (such as leading to changes in reproductive hormone levels and associated changes in fertility) have not been observed in GalSafe® pigs.

The growth of GalSafe® pigs through multiple generations has been determined to be similar to that generally observed in comparators without the IGA. Live growth demonstrated that GalSafe® pigs grow in a manner that is not different from comparators without the IGA. Weight at reference ages (such as birth, weaning, etc.) and average daily gains were normal when compared to comparator animals without the IGA. Live animal growth for GalSafe® pigs falls predominantly within the normal range that has been established from mathematical growth models from birth to physiologic maturity

for comparators without the IGA. Furthermore, a second level of growth, skeletal growth, assessed by an evaluation of long bones, did not demonstrate differences in macroscopic or microscopic bone characteristics when compared to comparator animals without the IGA. Skeletal growth demonstrated that these tissues were physiologically and anatomically normal, and fit previously established allometric skeletal growth models for comparators without the IGA. Histology confirmed that changes in bone morphology with age are similar in appearance to published descriptions of bone histology from comparators without the IGA of comparable age.

There are no detectable differences in health status between GalSafe® pigs and comparators without the IGA. A retrospective review of treatment records revealed that GalSafe® pigs are susceptible to the same illnesses and diseases as comparators without the IGA. Notably, the overall prevalence of diseases and illnesses is lower in the GalSafe® herd relative to comparators without the IGA; this is most likely due to the physical containment and husbandry practices that are employed to manage the GalSafe® herd. After treatment for illnesses (by medicines typically administered to comparators without the IGA for similar illnesses), the GalSafe® pigs were found to respond to treatment the same as comparators without the IGA. Piglet morbidity from GalSafe® sows is similar to published reports derived from comparators without the IGA. Post weaning morbidity appears to be lower, again most likely related to the management practices used to sustain the herd. Additionally, a thorough evaluation of the physiological status of healthy GalSafe® pigs that included necropsy, hematology, and serum chemistry evaluations did not reveal any aberrant anatomy or any evidence to suggest the presence of pathology. Thus, these evaluations indicated GalSafe® pigs possess normal pig anatomy, as well as normal hematology and serum chemistry parameters.

No differences have been observed in the reproductive systems or functions of GalSafe® pigs compared with comparators without the IGA. The reproductive anatomy of the GalSafe® pig was evaluated and found to be the same in appearance and function to comparators without the IGA. Major reproductive events in the reproductive cycle, specifically weaning, puberty, estrus (onset and duration), and gestation, occurred at similar timeframes when compared to comparators without the IGA. GalSafe® pigs exhibited the same behavior during breeding and farrowing that is observed for comparators without the IGA. Quantitative traits that were defined to be number of teats, gestation length, and litter size were demonstrated to be consistently normal when compared to that of comparators without the IGA. Notably, the birth of live piglets as a result of GalSafe® sow and boar matings, under normal husbandry and management conditions, to sustain the GalSafe® line for at least

eleven (11) generations further substantiates the GalSafe® reproductive system is functional and normal.

Therefore, with the exception of the intended absence of alpha-gal, there are no detectable phenotypic differences between GalSafe® pigs and comparators without the IGA.

2.5 Genotypic and phenotypic durability

Comprehensive analysis of the genomes from the lineage progenitors to the F11 generation of GalSafe® pigs confirmed that the targeted insertion is stably transmitted to progeny through normal breeding and conformed to Mendelian inheritance. Additionally, phenotypic traits of growth, health, and reproduction were found to be similar to that of comparators without the IGA through multiple generations. Concomitantly, the intended design, lack of detectable alpha-gal, is stable, persistent and durable regardless of gender, age, and generation from lineage progenitors.

Revivacor has established genotypic and phenotypic durability plans for post market record keeping and reporting to ensure that GalSafe® pigs that are maintained by Revivacor are equivalent to those evaluated for safety and effectiveness during the review process. Adherence to the plans during routine production of GalSafe® pigs is intended to ensure that no new hazards are introduced during the production of these pigs.

2.6 Effectiveness/Claim validation

The effectiveness of the IGA in homozygous GalSafe® pigs (i.e., lack of detectable alpha-gal) was evaluated by three different complementary methods at the cellular level, the tissue level, and the whole body level. Alpha-gal was persistently and durably undetectable regardless of gender, age, and generation.

Section 3.0 GalSafe® herd management: production, containment, and disposal

3.1 Production plan

The GalSafe® herd contains pigs without the IGA, hemizygous GalSafe® and homozygous GalSafe® pigs. The herd is divided into the breeding herd and the production herd. Currently, the herd is small (<100 pigs); when appropriate, the animal facility may be expanded to accommodate a herd that is up to 10 times its current capacity (1000 animals total).

Pigs selected for the breeding herd are intended for the reproduction of progeny to replace retired breeders, and to sustain or to increase the scale of the herd. The current breeding herd makes up about ~8-10% of the total herd at a ratio of one boar per five sows (gilts). The breeding herd is a group of not only homozygous GalSafe® pigs, but may also include hemizygous GalSafe® pigs and pigs without the IGA. Dams will be group housed and intact boars will be kept in individual pens.

The intended use of homozygous GalSafe® pigs in the production herd are as sources of food products for human consumption or as sources of human therapeutics including excipients, devices, drugs, or biological products. It is anticipated that most of the homozygous GalSafe® pigs will be designated to the production herd. Weaned pigs designated to the production herd are approximately 50% castrated barrows (males) and 50% juvenile gilts (unbred females) that are euthanized around 6-7 months of age.

The goal of the production plan is to efficiently maintain or expand the number of GalSafe® pigs produced at the facility up to 1,000 animals to accommodate demand for human food and therapeutics, while ensuring herd hybrid vigor (outbreeding enhancement). This goal will be met via continuous application of line breeding and periodic outcrossing. Individual breeder pairs will be matched only from pigs that are accepted into the breeding herd. Specific procedures are followed as described in applicable standard operating procedures (SOPs) and include the completion of breeding records identifying the pedigree of any progeny produced.

3.2 Animal Housing Facility in Iowa

3.2.1 Animal welfare

The facility and staff maintain high standards of animal welfare during the entire lifespan of the pig. The facility is registered with the USDA and is routinely inspected by Animal and Plant Health Inspection Service (APHIS) under the statutory authority of the Animal Welfare Act. The facility where the GalSafe® pigs are housed has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) since the first pigs with the IGA were located on the facility and is reaccredited every 3 years. The most recent documents associated with inspection and accreditation are maintained on file at the facility.

3.2.2 Location

The facility is located in rural northern Iowa on approximately 12 acres of land. The site is surrounded by mature trees and sits off the closest public road approximately a quarter of a mile. The

surrounding area is primarily semi-rural hay and crop land. The area contains scattered residences and a few isolated sub-divisions. There are no wildlife preserves or parks (federal, state, county or city) adjacent to or within a 20 mile radius of the facility. There are no agricultural swine operations on adjacent properties.

The site was chosen as suitable based on its location away from any activities on abutting properties that would pose an environmental hazard, the suitability of the terrain for agricultural operations, no significant environmental risks on or close to the property, and access to water that meets National Primary Drinking Water Standards (NPDWS).

3.2.3 Description of facility

All pigs housed in the facility are separated from the surrounding environment by a minimum of three physical barriers. The facility is surrounded by a perimeter fence (tertiary physical barrier) that is topped with barbed wire. There are lockable gates on this perimeter fence line to allow entrance and egress to the facility. The pigs are housed inside a fully enclosed building (secondary physical barrier) that is partitioned into various sized pens (primary physical barrier). The barriers are designed to ensure that animals cannot escape from containment. Specific details of the physical containment are provided in Section 3.2.4.

The site is separated into non-biosecure and biosecure areas. Biosecure areas[§] are designated as such with respect to preventative measures to reduce the risk of introduction or transmission of diseases, and not necessarily escape of the pigs, although it does have the effect of providing physical confinement. The non-biosecure area includes the parking lot for employee vehicles and everything outside of the perimeter fence. The biosecure area of the property is the area that is designated for the housing and care of animals and is clearly defined and contained by the chain link perimeter fence.

The location of all buildings and pens, breeding and farrowing areas, and the tissue procurement room is within the biosecure areas of the facility. Most buildings are connected by enclosed corridors. Specific procedures for movement of pigs in and between buildings are followed, as described in applicable SOPs and documented on appropriate records. Fresh air is provided to each building via mechanical ventilation through DOP95 filters. Building temperature is maintained at approximately 60°

[§] Biosecure areas as defined by Revivacor, and used hereafter in this EA, are defined as the areas inside the perimeter fence of the farm facilities including the buildings that are part of the perimeter.

to 80°F. Indoor lighting is provided for approximately a 12 hour period every day by waterproof fluorescent lights.

An integrated pest management program is maintained at the facility. Each building unit is surrounded by gravel or concrete as a buffer zone to prevent the access of rodents to the facility. Additionally, a rodent control program from a third party is established at the facility that consists of rodent traps, bait stations (poison), and insecticides. All buildings are fully enclosed with ventilation provided through DOP95 filters to prevent access of birds and other small animals to the interior of the buildings that house the pigs. Pest management activity is recorded in a logbook that is maintained at the facility.

3.2.4 Physical containment

All pigs are primarily contained in the animal housing facility that has at least three physical barriers separating the pigs from the outside environment. The pigs have been managed in the facility for over nine years and there has never been an escape or breach in containment.

3.2.4.(1) Pens (primary physical barrier)

Pigs are contained in pens (primary physical barrier) that are designed to conform to the layout of each building. Pens are of various sizes to accommodate pigs of various ages and meet or exceed accepted industry practices for housing pigs. Pens consist of panels and gates constructed of either steel tubing with a galvanized or painted finish (Table 1). For younger animals, PVC fencing panels (≈1 ½ inch thick) with stainless steel vertical supports are used (Table 1). The height of the pens extends to near the floor and are appropriate for the age or size of the pig (Table 1). The spaces between the square tubing are sized appropriately to contain the animals housed inside. One or more sides of the pen may consist of the interior of the building wall (¾ inch thick plywood attached to standard wood framing and a ⅛ inch thick fiberglass surface coating). Vertical posts are secured to the concrete or slatted flooring or wall studs via lag bolts, j-bolts or similar. Renovations, reconfigurations, or new construction will follow a similar design.

Table 1. Description of pens for the different ages of pigs.

Type of animal	Material	Height
Older animals (>5 months)	◆ 1 inch x 1 inch square steel tubing finished with paint or	≈3-4 feet

	◆ 3/8 inch galvanized steel rod	
Younger animal (1-7 months)	◆ 1 ½ inch thick PVC panels with stainless steel vertical posts (1.5 inch “C” channel)	≈3 feet

3.2.4.(2) Buildings and corridors (secondary physical barrier)

The main building is the controlled access entrance for people and supplies. It contains a reception area, shower, and office space. The lock on the main entrance is unlocked during normal business hours to allow access for shipping/receiving and vendors. This only allows access to the reception area and no further. Doorways to lockers and shower facilities are within the reception area; these doors are locked at all times and require a key for access in order to enter the remaining portions of the building, including pens that house pigs as well as other buildings in the biosecure area.

All pigs are kept inside enclosed buildings at all times (secondary physical barrier). When moving between breeding, farrowing and finishing pens, pigs move through the building via corridors and are always accompanied by farm personnel. If pigs are being moved between buildings with unenclosed corridors, the pigs are always accompanied by farm personnel, contained inside a crate in the exiting building and released from the crate after arrival into the incoming building. Interbuilding movement of pigs occurs infrequently and typically at a major life stage (e.g., farrowing, weaning, breeding, tissue procurement, etc.). Specific procedures for movement of pigs in and between buildings are followed as described in applicable SOPs and documented on appropriate records. The tissue procurement facility is within the biosecure area of the facility. All building doors that allow access to outside the facility are kept closed except when site personnel need to enter or leave the building. These procedures are designed to minimize the risk of a pig escaping the building.

Buildings are constructed with concrete foundation and floor, wood frame walls and rafters. The interior finish consists of fiberglass laminated plywood as well as solid plastic sheeting. The exterior of the building including the roof is painted steel. Additionally, smaller animals may be housed in a modular structure that consists of a steel and wood frame with an interior surface that is seamless fiberglass and an exterior that is painted steel. Any renovations or new construction within the facility will follow a similar design.

3.2.4.(3) Perimeter fence and gates (tertiary physical barrier)

The perimeter fence (tertiary physical barrier) is constructed of chain link fencing six feet tall that is topped with three strands of barbed wire (≈ 1 ft). If not traversing finished concrete pathways, the fence is buried below grade. In order to keep the fence line level with the horizon and to account for variation in topography, the fence is buried to a depth of ~ 4 -8 inches. Regardless, the height of the fence (ground to the top strand of barbed wire) exceeds six feet around the entire perimeter. The fence is secured to vertical steel posts (≈ 1.5 inch outside diameter) that are spaced approximately 10 feet apart. At each gate, there is a poured concrete apron, which minimizes the space under the gate and prevents digging. This perimeter fence is designed to prevent outside intruders from entering the biosecure area as well as to keep pigs housed in the facility from escaping.

All access gates located along this perimeter fence are locked at all times except when ingress or egress is required. The personnel that can open the locked gates have been trained in the SOPs related to security of the perimeter fence.

3.2.5 Procedural containment

There are multiple procedural containment measures in place at the facility to reduce the likelihood of escape of all pigs that are housed there. These are described in depth below and include personnel training, controlled access, security procedures and a disaster guidelines.

3.2.5.(1) Personnel and training

The farm staff consist of a relatively few people who are screened via a background check prior to hiring. Employees are trained on the relevant programs (e.g., biosecurity) and SOPs (e.g., visitor policy) commensurate with their job descriptions. This minimizes the risk of unauthorized people having site access and ensures GalSafe® pigs are properly contained.

All personnel enter the facility through the main entrance. Street shoes are removed before entry into the locker rooms. All staff members must shower “in” and change into facility dedicated attire before entering into the biosecure area. Area-dedicated rubber boots or boot covers, coveralls and gloves are worn at all times when in individual animal pens or buildings. These items are put on at defined lines (door way, gate, etc.) as the staff member enters the area and removed at the same line as the staff member leaves the area. The specific procedures are described in applicable SOPs.

Employees wear dedicated clothing that is freshly laundered on a daily basis. If clothing becomes overly soiled, employees are expected to change into freshly laundered clothing during their work shift. Disposable gloves and other personal protective equipment are worn when working with animals

(bleeding, treatment, etc.). Hands are washed regularly throughout the day to minimize the passing of microbes or microbial flora from one animal to another.

Personnel at the facility account for the presence of all pigs on a daily basis, and document via weekly inventory records and monthly inventory reports, as described in the SOPs. Personnel are present at the facility seven days a week and are instructed to monitor the status of the pigs and facilities.

3.2.5.(2) Staff and visitor access

All staff and visitors must enter the building through the primary access door. Visitors must register via signature prior to gaining further admittance to the building. All visitors must fill out a detailed biosecurity questionnaire, and be approved for entrance beyond the office area. Site access may be restricted or denied by management depending on the visitor's biosecurity risk**. No one enters the facility at any time without showering and changing into site-dedicated clothing and footwear. Visitors are chaperoned during the visit; contractors are escorted to and from their work site. The specific procedures are described in applicable SOPs.

3.2.5.(3) Facility security

Significant effort has been directed to prevent criminal or malicious intent to the facility or the GalSafe® pigs. The facility is located in a remote region of the country that is over 100 miles from the nearest large metropolitan area. It blends into the surrounding environs due to presence of mature trees around the perimeter and sits off the road approximately a quarter mile. The area surrounding the facility is semi-rural hay and crop land with a few scattered residences. The primary access road is patrolled by county and city law enforcement personnel. The facility is surrounded by a perimeter fence as described previously (Section 3.2.4.(3)); all pigs there are contained within redundant physical barriers (Section 3.2.4). There are signs posted on entry gates that direct visitors to the main entrance and indicate the biosecure perimeter. Access to the facility is only through a controlled main entrance doorway that is locked at all times except during normal business hours; this entrance only allows access to a small reception area as described (Section 3.2.4.(2)). The only vehicles allowed within the biosecure area are those essential for servicing the farm as there are already dedicated vehicles on the farm for movement between the buildings on the site. The farm is staffed with qualified personnel (Section

** Per the SOP, biosecurity is defined as the practices and protocols designed to prevent the transmission of disease causing agents from one area or population, to another.

3.2.5.(1)) during normal business hours and during weekends and holidays. All buildings, including gates, doors, and pens are checked daily including weekends and holidays by farm personnel. Any suspicious activity is reported to management or, if appropriate, to local law enforcement officers.

3.2.5.(4) Disaster guidelines

Disaster guidelines have been developed to ensure welfare of all pigs housed at the described facility during a disaster where power and water may be disrupted. The plans include animal evacuation (if necessary) as well as stockpiling supplies for staff as well as animals that includes food, feed, and medicine. Importantly, the plan identifies an internal communication center and procedures for reporting a breach in containment at the facility. The specific procedures are described in applicable SOPs.

3.2.6 Animal tracking

3.2.6.(1) Individual pig identification

At farrowing, each piglet is identified with a unique animal identification (ID) that is maintained throughout the life of the pig, and ensures traceability of any pig or derivatives through all operational areas. The unique ID is recorded on written documents and placed directly on the pig via ear notch and via a tag. After completion of a durability assessment, an additional identifier is placed on the pig that contains pre-defined symbols that correspond to the pig's genotypic identity. In short, each pig is identified with "G0" (pig without the IGAs), "G1" (hemizygous GalSafe®), or "G2" (homozygous GalSafe®). Applicable records reference the unique ID through all operational areas in order to determine the complete production history of the pig including the ultimate disposition for any pig in the GalSafe® herd. Specific procedures for identifying and maintaining pig identification are described on applicable SOPs that are maintained at the facility. The method of identification is maintained throughout the life of the pig up through euthanasia or slaughter.

3.2.6.(2) Daily observations

All buildings, including gates, doors and pens are checked at least once a day including weekends and holidays by qualified personnel to ensure barriers are secure. After each inspection, personnel annotate date, name, and the presence or absence of any findings. Any unusual events such as unsecured pens, pigs outside of pens, sick, or deceased pigs are immediately addressed. The specific

procedures are described in applicable SOPs and documented on applicable records that are maintained at the facility.

3.2.6.(3) Weekly and monthly inventory reports

Weekly inventories are documented by taking a physical head count of all animals in the herd, including GalSafe® pigs. This physical head count is reconciled with the current inventory record in the barn, as well as the number of additions and subtractions in the herd during the week. If any discrepancies in inventory occur they are communicated to management for resolution. The specific procedures are described in applicable SOPs and documented on applicable records that are maintained at the facility.

Monthly inventory reports are provided to Revivacor that identify current animal inventory, as well as any additions (births) or subtractions (transfer, death, and tissue harvesting) from the previous month. Any shipments of tissues are also noted on this monthly inventory report.

3.2.7 Tissue procurement

The intended use of homozygous GalSafe® pigs in the production herd are as sources of food products for human consumption or as sources of human therapeutics including excipients, devices, drugs, or biological products. Derivatives originating from the homozygous GalSafe® pigs that may serve as a food product are muscle, liver, kidney, skin, bone, and fat and are obtained from a single abattoir described in Section 3.4. Examples of derivatives that may serve as a manufacturing intermediate include heart valves, blood vessels, dermis, tendon, and bone.

Homozygous GalSafe® pigs that are selected for collection of raw materials are euthanized using a method that is recommended by the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.⁷³ Specific procedures are followed as described in SOPs. A pig may be excluded for collection of raw materials by the attending veterinarian if it is noted to have aberrant anatomy or the presence of pathology. Post euthanasia, selected tissues are removed, packaged, labeled, preserved and shipped to Revivacor's collaborators/business partner(s) for subsequent use to produce human use medical products.

A separate tissue procurement room is located within the biosecure area of the facility where the GalSafe® herd is managed. Homozygous GalSafe® pigs are euthanized and tissues procured without the pig leaving the premises. Specific procedures are followed as described in SOPs that include completion of applicable records that are maintained on file at the facility. Animals and applicable derivatives are

tracked and labeled as described below. Unused derivatives from procurement are disposed as described in Section 3.2.8 and Section 3.4.4.

3.2.7.(1) Labeling, packaging, and shipping from Iowa facility

Manufacturing intermediates that are derived from the production facility in Iowa that are intended for further fabrication into a human therapeutic will be packaged in a manner appropriate for the design requirements of the final product. Products prepared for shipment will be transported (through prior arrangement) by a freight-forwarder. The freight-forwarder will arrange, manage, and monitor air-freight shipment of the product to Revivacor's business partner(s), where control will be assumed by Revivacor's collaborators/business partner(s).

3.2.8 Carcass and remnant disposal

All pig carcasses and any tissue remnants that are not designated to the abattoir or distributed to Revivacor's collaborators/business partners are disposed of as described in FDA's Guidance for Industry 187 (i.e., incineration or composting). Some carcasses and remnants are temporarily stored frozen until sufficient mass (~20 – 200 lbs) accumulates that necessitates disposal. Disposal of carcasses is completed using one of two different methods: incineration or composting. The particular disposal method(s) that is employed will be a function of the scale of the GalSafe® herd and operating costs. Specific procedures are followed as described in applicable SOPs, and the procedures include completion of appropriate records for each pig that exits the GalSafe® herd. All disposal records are maintained on file at the facility.

If there is a need for specialized post-mortem diagnosis, it may be necessary to transport the carcasses or tissue specimens to a specialized pathology laboratory. These specimens are carried or shipped in a leak proof container, i.e. plastic bag or tote. An agreement will be in place that ensures the disposal of carcasses and tissues as a biological hazard and will not enter the food or feed supply.

3.2.8.(1) Incineration

Carcasses and remnants may be incinerated using equipment that is located on the facility. All incinerators are operated according to the manufacturer's instructions to ensure that carcasses or remnants are properly reduced to ashes. Specific procedures are followed as described in applicable SOPs and include completion of appropriate records for each pig that exits the GalSafe® herd. All disposal records are maintained on file at the facility.

3.2.8.(2) Composting

Carcasses and remnants may be composted at a site inspected by the Iowa Department of Natural Resources (IDNR). Specific procedures are followed as described in applicable SOPs and include completion of appropriate records for each pig that exits the GalSafe® herd. All disposal records are maintained on file at the facility.

3.2.8.(3) Manure, urine, and other soluble waste disposal

All farm soluble waste is handled in a controlled manner, including that from GalSafe® pigs. These waste components include urine, feces, and other soluble waste products (placenta, blood, etc). All soluble waste is diverted to a holding pit where it is held for several days to several months, depending on capacity. It is then pumped to an on-site manure lagoon, where it is mixed with other farm manure waste.

Two types of excreta collection/flooring systems exist in the animal housing facilities. The excreta is in the form of a slurry that is a combination of manure, urine, and wash water which allows it to be pumped and/or drained as needed. Some of the flooring in the pig pens consists of slats made of concrete, cast iron, steel, or plastic. This allows for the excreta to fall through the flooring. Under the slats is a primary holding pit which is capable of containing several days' to several months' worth of excreta. This primary pit is emptied as needed due to space limitations or during times of cleaning and sanitization of the building. Other flooring in some of the pig pens is solid and consists of solid concrete with floor drains or grates in the room for the removal of waste. The excreta are removed daily from these areas using hand tools. The excreta are moved to the floor drain or grate and washed down the drain into a primary holding pit. This primary pit is emptied as needed. The specific procedures are described in applicable SOPs for waste disposal.

Lagoons are large volume, longer term storage of animal excreta and wash water. When primary holding pits are emptied into lagoons located on the facility, they are pumped through permanent pipes laid underground, or in some cases a temporary hose laid on the ground. All of the waste from multiple primary holding pits is emptied into one of two lagoons with a combined capacity of 1.1 million gallons. Waste remains in the lagoons for several days to several months depending on the season. The lagoons are pumped as needed, typically once or twice per year. The material from the lagoon is spread on commercial agricultural crop ground using modern techniques which include large tankers which "knife" the material below the surface of the field, to minimize odor and run-off. The location where the

material is spread is documented. These procedures are in accord with regulations of the IDNR, and the specific procedures are described in applicable farm facility SOPs for waste disposal.

3.3 Transportation of live pigs

Transportation of live pigs from the GalSafe® animal operation occurs by qualified personnel from the facility or by a qualified service provider. This may be performed for research, veterinary, biosecurity, or welfare concerns; or food processing. When transporting pigs intended for food use, only homozygous GalSafe® pigs designated for food use will be present on the trailer during transit.

Pigs are transferred (loaded onto a cart inside the biosecure area) from buildings at the housing facility to an enclosed livestock trailer that has internal gates (floor to ceiling) that can be configured appropriately to safely hold multiple animals. The optimal penning (1 or more areas inside the trailer) will be determined by personnel from the facility or by the service provider. All gates (internal and external) are secured with pin locks (or similar) to prevent movement or opening during transit. In the highly unlikely event that a GalSafe® pig escapes during transport, it will be reported to FDA-CVM.

A shipping invoice that contains each pig's ID and a description of the pig's genetic identity accompanies the shipment. After delivery, records of receipt are disseminated and archived for at least two years at the animal housing facility and at Revivacor.

Specific transportation procedures are described in applicable SOPs that are maintained by the animal housing facility.

3.3.1 Traceability of live GalSafe® pigs through final disposition

As previously described (Section 3.2.6.(1)), each piglet is identified with a unique animal ID that is maintained throughout the life of the pig and ensures traceability of any pig or derivatives to their ultimate destination, including the secure transportation of homozygous GalSafe® pigs to the identified abattoir. .

3.4 USDA inspected abattoir in South Dakota

Homozygous GalSafe® pigs intended for food use are shipped to a specified abattoir, otherwise known as a slaughterhouse, in southern South Dakota that is routinely inspected by the USDA. The USDA is the responsible regulatory authority for the homozygous GalSafe® pigs after receipt at the abattoir and prior to slaughter, and the associated meat products, including any packaging labels on meat products. In addition, all of these pigs are subject to inspection by USDA prior to slaughter. In brief, all phases of the abattoir operation conform to USDA's safety, wholesomeness, and labeling

standards per its enabling regulations codified in 9 CFR Chapter III USDA Food Safety and Inspection Service (FSIS), including relevant sections of subchapter A (Parts 300 to 381), subchapter D (parts 390-392), and subchapter E (parts 412 - 500). For this reason, a detailed description of the abattoir and distribution operation is not provided. A general overview of animal management and relevant USDA regulations, as they may be applied to homozygous GalSafe® pig management, is provided below.

3.4.1 Animal management

The abattoir processes no more than 15 animals per day that are typically scheduled by appointment several months in advance. Although the abattoir can house animals overnight, all homozygous GalSafe® pigs received and staged for USDA inspection prior to slaughter (antemortem) are intended to be slaughtered on the day of receipt. In brief, these pigs are unloaded in the early morning from the livestock trailer and moved into an antemortem inspection area via a cattle chute. The antemortem inspection area is located inside an enclosed barn and each pig is housed in steel holding pens that safely secure the animals. The antemortem inspection area has one external door to facilitate movement of human handlers that is secured when animals enter. Therefore, there are a minimum of two levels of containment at the abattoir.

3.4.2 Packaging, labeling, and storage of GalSafe® food products

All homozygous GalSafe® food products will be packaged in containers and properly labeled per adherence to 9 CFR Part 317. Storage parameters for each packaged product will be appropriate to ensure safety and wholesomeness for human consumption. Labels and labeling will adhere to USDA requirements. Regardless of destination, shipping and transportation will be performed in a manner to ensure safety and wholesomeness for human consumption.

3.4.3 Procurement of manufacturing intermediates at the abattoir

Selected materials from homozygous GalSafe® pigs may be designated as manufacturing intermediates intended for further fabrication into a human use non-food product such as a medical product by Revivacor's business partner(s). Manufacturing intermediates will be collected via a specimen permit (9 CFR 314.9) and procured, preserved, and shipped per written instructions from Revivacor's collaborators/business partners.

3.4.4 Disposal of pig carcasses, waste and other remnants at the abattoir

All homozygous GalSafe® pig carcasses and any remnants that are deemed edible but not processed into food will be properly disposed via rendering per 9 CFR Part 315. Animals, carcasses or derivatives therefrom that are disqualified for processing or other reasons will be properly disposed of per 9 CFR Part 314. All the disqualified materials are placed in large storage containers clearly labeled for rendering. These storage containers and other equipment used for rendering are kept in separate areas away from other areas where edible products are processed and stored. The contents of the containers labeled for rendering are effectively destroyed via the addition of a denaturant per 9 CFR 325.13(b) to give the rendered fat so distinctive a color, odor, or taste that it cannot be confused with an article of human food. Rendered products can be used in animal feed, ingredients for industrial products (e.g., fatty acids, lubricants, plastics, printing inks, explosives), and consumer products (e.g., soap, cosmetics, shaving cream, perfumes, polishes, paints, calking compounds).^{74, 75} Wastewater from the abattoir, which includes manure, is regulated by the Environmental Protection Agency (EPA) under the Meat and Poultry Products (MPP) Effluent Guidelines and Standards per 40 CFR Part 432.

Section 4.0 Affected environment

This EA evaluates the production of GalSafe® pigs at a single facility in northern Iowa and holding and slaughter of homozygous GalSafe® pigs at a single abattoir in southern South Dakota. The animal housing facility and abattoir, while in separate states, are less than 20 miles apart; therefore, the affected environment is similar at both facilities. Production of GalSafe® pigs will occur within a highly-contained facility described previously (Section 3.2). Slaughter will occur at the USDA inspected abattoir described previously (Section 3.4).

4.1 Location and climate

“The State of Iowa comprises 56,288 square miles, primarily of rolling prairie, located in the middle latitudes between the Mississippi and Missouri rivers. The interior continental location is approximately 850 miles north of the Gulf of Mexico, 1450 miles east and northeast from the Pacific Ocean, 1050 miles west and northwest from the Atlantic Ocean and 1050 miles south-southwest of Hudson Bay. The extreme north-south distance across Iowa is 215 miles; the extreme east-west distance, 330 miles. Elevation changes are small across the State, varying from a maximum of 1670 feet in Osceola County in northwestern Iowa to 480 feet at the southeast tip of the State at the confluence of the Des Moines and Mississippi rivers.”⁷⁶

Iowa's climate, because of its latitude and interior continental location, is characterized by marked seasonal variations. During the six warmer months of the year, the prevailing moist southerly flow from the Gulf of Mexico produces a summer rainfall maximum. The prevailing northwesterly flow of dry Canadian air in the winter causes this season to be cold and relatively dry. At intervals throughout the year, air masses from the Pacific Ocean move across the western United States and reach Iowa producing comparatively mild and dry weather. The warm autumnal days are a result of the dominance of these modified Pacific air masses. Hot, dry winds, originating in the Desert Southwest, occasionally reach into Iowa during the summer producing unusually high temperatures and crop desiccation.

In general, the climate in Iowa ranges from dry in the winter months to wet in the spring and summer months. Average yearly rainfall is documented to be 26 inches and an average yearly snowfall of 32 inches; average temperature in the coldest month, January, is between 5 to 25°F while the hottest month, July, ranges between 61 to 86°F.⁷⁶ The nearest location for which climate data is available at the production facility is tabulated by month for average minimum, average maximum daily temperatures as well as average precipitation values (Table 2).⁷⁷

Table 2. Average monthly high, low and rainfall values between 1981 and 2010 for the region where the production facility is located.⁷⁷

	Month	Jan	Feb	Mar	April	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Temperature	Average High (°F)	25	31	44	61	73	82	86	83	75	63	44	29
Temperature	Average Low (°F)	5	11	23	35	47	57	61	58	49	37	24	10
Precipitation	Average (inch)	0.6	0.7	1.9	2.5	3.4	4.4	3.5	3.3	3.0	2.0	1.1	0.8

4.2 Landscape and carnivorous species

According to the IDNR website, "Iowa's population grew rapidly with increased riverboat traffic up the Mississippi and the beginning of a railroad in 1853. Demand for land and natural resources increased. Ninety-five percent of Iowa's wetlands were drained or filled in, 70 percent of Iowa's forests were cleared, and 99.5 percent of Iowa's prairies were plowed within a 100-year period."⁷⁸

"Many habitats that once supported an abundance of wildlife species were converted to cropland, towns, railroads, and cities. It is estimated that only one tenth of the state is similar to what the first settlers found. These remaining areas are small, widely scattered remnants—compared to

millions of acres of contiguous habitat that once existed. Animals that need large expanses of habitat (e.g., large predators) may be gone from Iowa forever. Others have adapted and survived.”⁷⁸ IDNR provides substantial descriptions of extirpated, endangered, expanding, and reintroduced species.⁷⁸

“Iowa carnivores include mink, weasel, badger, coyote and otter. Some large carnivores are finding a home in Iowa. Sightings of black bear, mountain lion, bobcat, and wolf are more frequent each year. Recent bear, mountain lion, and bobcat sightings have included young of the year, indicating reproduction in the wild.”⁷⁸ Coyotes in particular are prevalent in Iowa and are proficient predators, possessing the speed, strength, and endurance necessary to tackle prey as large as adult deer.^{79, 80}

4.3 Prevalence of feral pig populations in the United States and affected environment

The pig, *Sus scrofa*, is a species that is presumed to have been domesticated between 8,000 – 10,000 years ago.^{81, 82} Genetic evaluation has revealed “multiple centers of independent pig domestication.”^{81, 82} Domesticated pigs are an efficient agricultural endeavor as they both complement and supplement other agricultural operations such as converting inedible feed products into valuable food products, and their waste maintains soil fertility.⁸³ Currently, there are more than 70 breeds⁸⁴ of domestic pigs in the world with various differences in appearance and geographical location - such breeds as Yorkshire, Duroc, Hampshire, Chester White, Spot, and Landrace, are commonly used for breeding in the United States.⁸⁵⁻⁸⁷ Domesticated pigs adapt well to a wide variety of climates, facilities, and diets.⁸⁸ Currently, almost all domesticated pigs produced in the United States for food are housed indoors, with feed, water and air quality strictly controlled, and biosecurity measures in place to maintain the health of the herd.^{89, 90} They can breed year round.⁹¹ Generally, gilts are bred when they first come into estrus at about 7-8 months of age⁹¹ and duration of gestation is typically 114 days.⁹¹ Piglets are weaned about three weeks after birth^{91, 92}, and reach market weight at about 250 lbs^{83, 93} (≈6 months of age). Sows and boars retained for reproductive purposes generally can be used for multiple breedings.^{91, 94}

Pigs, specifically *Sus scrofa*, are not indigenous to North America.^{81, 95} Domesticated swine were introduced to the continental United States in the 1500s when the first Spanish explorers arrived in Florida and Texas.⁹⁵ Given their adaptability and ability to forage for their own food, they were popular livestock for settlers as they could be sustained primarily by free-range husbandry practices that persisted until the 1960s.^{81, 82} Many free-ranging domesticated pigs escaped and subsequently became feral.^{81, 95} Feral swine were documented around Spanish, French, and English settlements as early as the

16th and 17th centuries.⁹⁵ Additionally, Eurasian wild boars that were captured in their wild native European habitats were intentionally released on private hunting preserves in several areas of the continental US in the late 19th and early 20th century.^{81, 95} Although these hunting preserves constructed fences to contain the animals, these structures were inadequate or fell into disrepair.^{81, 95} Subsequently, the animals were free to forage beyond the preserves and have propagated with feral domestic pigs to produce progeny referred to as hybrids.⁹⁵

By the early 1980s, feral pigs ranged from the Coastal Plain of Virginia south to Florida, and west to Texas and California. The range of feral pigs appears to be continually expanding and the South remains the epicenter of feral pig populations.⁸² The Southeastern Cooperative Wildlife Disease Study (SCWDS) routinely surveys all state authorities regarding populations of established feral herds and publishes location of existing herds on its website.⁹⁶ Historical and current maps (Figure 2) are also provided and the range of feral pigs has expanded overtime.^{96, 97} Introduction to new areas is attributed to illegal translocation from established areas to new areas for sport hunting and escape from poorly constructed hunting preserves.^{98, 99} Once introduced, feral pigs are hearty; they have significant reproductive potential and adapt to a wide range of habitats.^{82, 100}

Feral boars reach puberty at a later age than domesticated conspecifics.¹⁰¹ Although feral boars may attempt to breed at 6 months, due to their smaller size, they are usually not successful until 12-18 months of age.¹⁰¹ Feral gilts reach breeding age between 6 to 12 months; breeding age appears to depend on the local environment as well as nutritional status.¹⁰¹ Gestation period is similar to domesticated sows and averages between 112 to 120 days.¹⁰¹ Neonatal litter sizes of feral pigs are smaller than domesticated swine and have been reported to be between 4 to 6 piglets.¹⁰¹ Some studies report wild-living swine breed seasonally, unlike domestic pigs which breed year round.^{82, 101} Piglets from feral dams have high mortality rates with some reports indicating 80% mortality within the first year of life.¹⁰² Herds or sounders consist of a small number of females and their young.^{82, 103} Feral pigs may travel together over a limited home range ($\approx 10 \text{ km}^2$), and do not migrate.¹⁰³ Mature males leave the group and live as isolated individuals.^{82, 103}

Feral pigs are known to display site fidelity, a type of philopatry^{††} that is manifested by the pigs remaining or returning to the geographic area that they were raised. Studies conducted on feral pigs on Ossabaw Island, GA indicated hand-raised piglets persistently returned to their homes after repeated

^{††} The tendency of an organism to stay in or habitually return to a particular area

attempts to relocate them in remote areas of the island. Similarly, after capture and relocation to the farthest points on the island (~5-10 miles), feral adults returned to their familiar range. Other studies report that pen-raised European wild hogs would not remain on managed wildlife areas but continually associated with more familiar farm habitats.¹⁰⁴ These pigs were repeatedly captured on agricultural lands and returned to the wildlife areas (22 attempts to relocate over a two year period).¹⁰⁴ The report indicates that they became so accustomed to wildlife personnel and vehicles that were dispatched to gather them that they would “jump into the truck bed” for recapture and “were tamer than any domestic hogs.”¹⁰⁴ Other reports indicate that even established feral herds that have agricultural association with humans are approachable and more easily captured or dispatched.⁹⁸

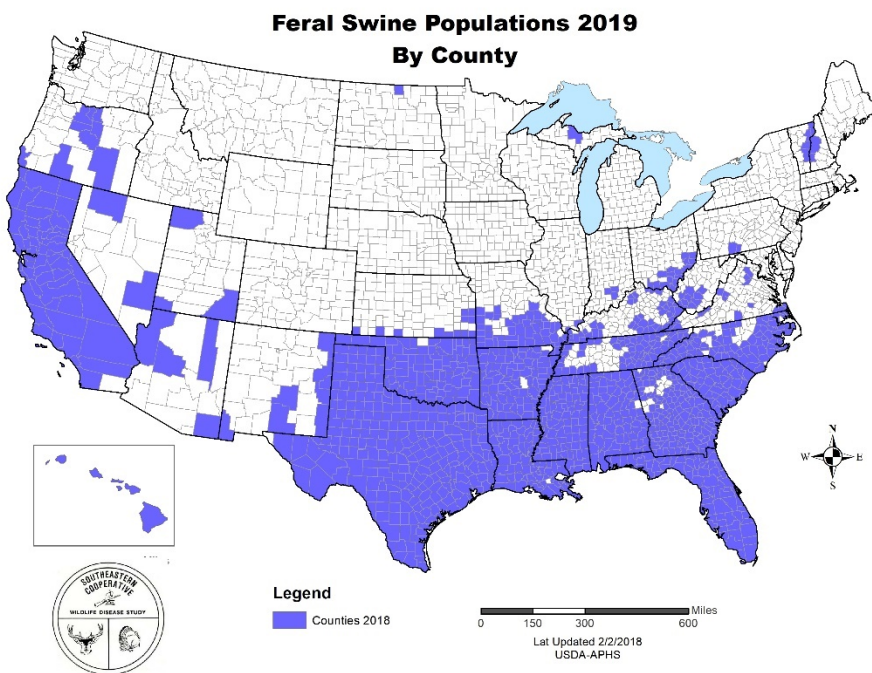


Figure 2. Location of established feral swine (blue marks) in the United States as of 2019.¹⁰⁵ There are no established populations of feral swine in Iowa or the border states of Minnesota, or South Dakota. However, there are a few scattered reports in Kansas, Nebraska, Illinois, and southern Missouri.

Based on the information above, wild living feral swine in the continental United States are primarily concentrated in the Southeastern United States, Texas and California. There are no established populations of wild living feral pigs in Iowa or the border states of Minnesota or South Dakota. However, there are a few scattered reports of established wild living feral herds in Kansas, Illinois, Nebraska, and southern Missouri (Figure 2).¹⁰⁵

Section 5.0 Risk characterization and analysis of impacts

5.1 Risk-related questions

The potential environmental impacts considered in this EA center on: 1) the likelihood and consequences of GalSafe® pigs, including hemizygous and homozygous GalSafe® pigs, escaping and becoming established in the environment, and 2) the likelihood and consequences of release of the *nptII* gene and NPTII protein into the environment. These risks are discussed and evaluated in the following sections, taking into account a series of risk-related questions identified below. Subsequent to this discussion is a summary of conclusions regarding risk and environmental impacts.

- What is the likelihood that GalSafe® pigs will escape the conditions of confinement?
- What is the likelihood that GalSafe® pigs will survive and disperse if they escape the conditions of confinement?
- What is the likelihood that GalSafe® pigs will reproduce and establish if they escape the conditions of confinement?
- What are the likely consequences to, or effects on, the environment should the GalSafe® pigs escape the conditions of confinement?

In addition,

- What is the risk from toxicity due to the presence of the *nptII* gene and NPTII protein to the affected environment?
- What is the likelihood of increased antimicrobial resistance in the affected environment occurring due to the possible presence of the *nptII* gene and the NPTII protein in manure and other waste products of GalSafe® pigs?

5.2 Risk of pigs with the IGA establishing populations in the affected environment

The risk assessment paradigm involves the integration of the probability of exposure with the probability of harm resulting from exposure. In evaluating the environmental concerns associated with genetically engineered animals and products of biotechnology^{††}, the National Research Council (NRC)¹⁰⁶ stated that exposure must constitute more than release or escape in order to pose a risk. The NRC defined exposure, more specifically, as the establishment of a genetically engineered animal in the

^{††} CVM currently uses the term “animals with the intentional genomic alteration (IGA)”

receiving community. The NRC also identified the following three variables as being important in determining the likelihood of establishment and determining the level of concern: (1) the effect of the transgene on the fitness of the animal for the ecosystem into which it is released; (2) the ability of the genetically engineered animal to escape and disperse into diverse communities; and, (3) the stability and resiliency of the receiving community. The components of fitness include all of the attributes of phenotype that affect survival and reproduction. For example, a transgene could improve the adaptability of an organism to a wider range of environmental conditions, or allow it to obtain nutrition from previously indigestible sources. A stable receiving community has an ecological structure and function that is able to return to the initial equilibrium following a perturbation; resiliency is a measure of how fast that equilibrium is re-attained.¹⁰⁷ The overall concern is a product of these three variables, not the sum; thus, if the risk associated with any one of the variables is negligible, the overall concern would be low (but not negligible).¹⁰⁶ These factors are considered when evaluating the risk questions in the following discussion.

5.2.1 What is the likelihood that GalSafe® pigs will escape the conditions of confinement?

The National Research Council (NRC) report¹⁰⁶ states that pigs have a moderate ability to escape confinement typical to commercial swine farms in the United States. However, GalSafe® pig production is carried out within a single facility with redundant measures (three physical barriers) to contain these pigs as previously described (Section 3.2.4). The pigs have daily contact with their human handlers and associate with humans similar to other domesticated pigs (Section 3.2.5). Additionally, significant procedural barriers are in place to ensure that staff are properly trained and maintain compliance to physical and procedural containment of GalSafe® pigs as previously described (Section 3.2.5). These attributes suggest that it is highly unlikely that GalSafe® pigs would be able to escape from the biosecure areas of the facility. In fact, these pigs have been managed in the facility for over nine years and there has never been an escape or breach in containment.

The possibility of unintended release, including from malicious activities and natural disasters, is highly unlikely. With regard to malicious activities, as previously described, there are controls in place to protect against these occurrences including employee background checks as well as limited and controlled access to the facility. With regard to natural disasters (e.g., tornados), in the event of an unintended release, there are disaster guidelines in place to aid in the recovery of GalSafe® pigs, as previously described.

During transport to the abattoir, the homozygous GalSafe® pigs are held in an enclosed livestock trailer that has internal gates (floor to ceiling) that can be configured appropriately to safely hold multiple animals. All gates (internal and external) are secured with pin locks (or similar) to prevent movement or opening during transit.

Slaughter is carried out at a single abattoir in South Dakota with two levels of physical containment (barns and pens) (Section 3.4.1). The animals are typically held at the abattoir for less than 24 hours. In addition, USDA oversight at the facility prior to slaughter will further reduce the likelihood of escape.

Conclusions: Due to the multiple, redundant physical and procedural containment measures at the production facility in Iowa, the abattoir in South Dakota, and during transport, the likelihood of GalSafe® pigs escaping to the surrounding environment is extremely low. This includes unintentional release such as could occur through malicious activities and natural disasters.

5.2.2 What is the likelihood that GalSafe® pigs will survive and disperse if they escape the conditions of confinement?

In the unlikely event of escape, it is expected that farm personnel would quickly identify a missing pig(s) during daily checks conducted to ensure secure containment (e.g., unsecured pens, pigs outside of pens) or during weekly animal inventories (Section 3.2.5). If issues are identified during either of these checks, personnel report the issue immediately to management for resolution, including recovery. In addition, each pig contains a unique ID (Section 3.2.6) which would allow it to be identified and recovered following escape. As previously mentioned (Section 4.3), feral pigs are known to display site fidelity that is manifested by the pigs remaining or returning to the geographic area in which they were raised. Therefore, it is expected that any pigs that escape will remain close to the facility which will allow for rapid recovery.

If GalSafe® pigs were able to escape and avoid recapture, they would need an ecosystem that meets their needs for food and habitat in order to survive and disperse.¹⁰⁸ As previously stated in Section 4.3, feral pigs are hearty and can adapt to a wide range of habitats.^{82, 100} The region where the production facility and abattoir are located (northern Iowa and southern South Dakota) are known for harsh winters (Section 4.1) which would likely affect the ability of the pigs to survive and disperse due to a lack of food and below freezing temperatures (Table 2). After all, Iowa is the largest swine producing state in the United States and accounts for roughly 30% of all swine production in the United States¹⁰⁹, however there are no established feral pig populations in Iowa (Section 4.3, Figure 2). However, this

does not entirely eliminate the possibility of escaped GalSafe® pigs establishing a population in Iowa or South Dakota because there are some known established populations of feral pigs in northern regions of the United States that have similar extreme winter weather (e.g., North Dakota, New Hampshire, Vermont); based on Figure 2, it appears to be a rare occurrence.

If the GalSafe® pigs were able to survive the harsh winter climate of Iowa and South Dakota, they would be hunted by humans and other predators including coyotes, foxes, and birds of prey^{95, 110} (Section 4.2). Humans are the major predators of feral pigs, as they are popular game species for hunters.^{82, 110} State regulations encourage the extirpation of feral pigs, and feral hogs enjoy no legal protection in Iowa.¹¹¹ Hunters are encouraged to watch for feral pigs while hunting other species and to kill them. There are no bag limits, no closed season, and no restriction on weapons. It is legal to kill feral pigs on private property and on public lands where hunting is allowed.¹¹¹ Authorities from the state of Iowa collaborate with federal and other state agencies to prevent and continue surveillance for feral swine.⁹⁶ Citizens are encouraged to report feral swine and any sightings are aggressively investigated. Iowa Code of Law prohibits transporting feral swine into Iowa, breeding feral swine and possessing feral swine.¹¹² Feral pigs are also susceptible to a variety of diseases and parasites which could affect their survival.^{82, 113, 114}

It is also important to note that GalSafe® pigs are not known to have enhanced fitness when compared to comparator pigs without IGA. Homozygous GalSafe® pigs do not have detectable alpha-gal residues on their cell surfaces; this phenotypic trait is not likely to impart any known fitness advantage if introduced to a receiving community. Thus, neither survival nor dispersal would be expected to be enhanced compared to pigs without the IGA, whether domesticated or feral.

Conclusions: The information provided in this section suggests that there are adequate procedures in place to quickly identify and report escaped GalSafe® pigs, allowing for rapid recovery. If escaped GalSafe® pigs could not be recovered, they could possibly survive and disperse in the environment surrounding the Iowa facility and South Dakota abattoir. However, the likelihood of long term survival and dispersion is low due to the harsh winter climate, hunting by humans, and predation by carnivores. This conclusion is further supported by the lack of established feral pig populations in Iowa and South Dakota where swine production is high. In addition, the IGA is not expected to provide any improved fitness to survive in the Iowa or South Dakota environments compared to pigs without IGA. Therefore, any GalSafe® pigs that escape would have a very low likelihood of surviving and dispersing in the ecosystem.

5.2.3 What is the likelihood that GalSafe® pigs will reproduce and establish if they escape the conditions of confinement?

In the highly unlikely event of an escape and survival, the potential establishment of pigs with the IGA into the surrounding environment would depend upon how many escaped and survived, their fitness characteristics, and their reproductive potential. As stated above, likelihood of escape from the production facility in Iowa and the abattoir in South Dakota and survival in the surrounding environment is low. In addition, the IGA does not provide any known improved fitness characteristics with regards to survival and reproduction compared to pigs without the IGA.

As described in Section 4.3, there are currently no established feral pig populations in Iowa or South Dakota. Therefore, mature GalSafe® pigs escaping into the receiving environment adjacent to the facility would not encounter conspecifics^{§§} or even closely-related species with which to interbreed. The majority of male pigs will be castrated and unable to reproduce if they were to escape. It is expected that the GalSafe® breeding herd will consist of no more than 200 pigs, at a ratio of one intact boar per five sows; therefore, the number of intact boars will be low (approximately 30). . In addition, intact boars are held in individual pens, further reducing the likelihood of escape. In order for pigs with the IGA to establish a reproducing population in Iowa or South Dakota environment, at least one intact boar and one female would need to escape. Its also important to note that intact male boars will likely not be transported to the abattoir for slaughter, also reducing the likelihood of escape. However, even if a pair (or more) of pigs capable of reproduction were able to escape and subsequently reproduce, it is extremely unlikely that they or any of their offspring would be able to survive long-term and establish a population in the affected environment. As previously discussed, there are no populations of feral pigs in this area (i.e., Iowa and South Dakota) despite the abundance of swine production there, which supports that swine establishment there is highly unlikely or will not occur.

Pseudo-establishment, that is, quasi establishment of a population in the environment due to a series of continual or closely spaced escapes, but with no actual reproduction, is also very highly unlikely for the reasons discussed previously under risk question 1, which discussed the likelihood of escape. Escape from the production facility and abbatoir is very unlikely due to the redundant forms of physical and procedural containment. Thus the probability of a single escape is very low, and the probability of a

^{§§} A conspecific is defined as a plant or animal belonging to the same species.

series of escapes is many times lower, thus precluding the potential for pseudo-establishment and any potential resulting impacts.

Conclusions: Although it could be possible for escaped GalSafe® pigs to reproduce; based on the reasons discussed above, the likelihood of a male and female GalSafe® pig with reproductive capabilities escaping the single production facility in Iowa and single abattoir in South Dakota and reproducing in the affected environment is extremely low. Accordingly, and because of all the reasons previously discussed in Section 5.2, the subsequent likelihood of establishment is extremely low. This extremely low likelihood is supported by the lack of existing feral pig populations in these states, an absence that is significant given that Iowa is by far the largest producer of pigs in the United States.

5.2.4 What are the likely consequences to, or effects on, the environment should Galsafe® pigs escape the conditions of confinement?

In the unlikely event of an escape of GalSafe® pigs where there is dispersal and/or reproduction and establishment, the ultimate consequences (environmental effects) would not be expected to be any different than if domesticated pigs without the IGA escaped and became feral. Feral pigs are opportunistic omnivores, and in the wild can eat roots, grasses, tubers, shoots, acorns, fruits, berries, earthworms, amphibians, reptiles, eggs, birds, rodents, carrion, and even newborn fawns of the white-tailed deer.^{99, 100, 115} They forage for their food by rooting, which is often problematic⁸² as rooting destroys crops and pasture land, and in wilderness areas destroys local or endangered plant species, and may impact sensitive indigenous animal species such as birds and turtles.^{82, 95, 99, 100, 116} A secondary effect of rooting behavior is to make these areas more susceptible to erosion, as well as enhance the proliferation of exotic plant invasion.

It is important to note that there are no populations of feral pigs in either Iowa or South Dakota (i.e., in the vicinity of the production facility or abattoir); therefore, any escaped GalSafe® pigs will be unable to reproduce with or contribute their genes to conspecifics. However, even if this were the case, and there were conspecifics present in the vicinity, because the regulated article is a recessive genotype, this would limit the establishment of the targeted insertion into the receiving community (feral population). Homozygous GalSafe® pigs possess the targeted insertion on both alleles. Thus, in the unlikely event of an escape of a homozygous GalSafe® pig of breeding age, and a subsequent mating with a feral pig, the resulting litter would be composed of piglets with the targeted insertion on only one allele (i.e., these would be hemizygous pigs with the IGA). After several additional generations of breeding with conspecifics, the introduced recessive trait (e.g., lack of detectable alpha-gal on the cell

surface) is expected to, according to Mendelian genetics, be eliminated from the gene pool. Thus, no long-term changes to the genetics of feral pigs, even if they were present in the area, would be expected to occur.

The worst case scenario for consideration would therefore be the escape, survival, and reproduction of a pair (or more) of reproductively competent male and female GalSafe® pigs. If this scenario were to occur and a population(s) of pigs were to establish in the affected environment, the expected consequences could include the destruction of crops and other habitat changes, but these effects would be expected to be no different than if a population(s) of escaped pigs without the IGA were to establish in the area.

Conclusions: The potential consequence to the affected environment from the establishment of pigs with the IGA is the destruction of crops, pasture land and wilderness areas, which would secondarily impact the habitat of indigenous animals. The consequences would be no different than if domesticated pigs without the IGA escaped and became feral.

5.2.5 Conclusions on risk of pigs with the IGA causing harm to the affected environment

The risk to the affected environment from animals with an IGA depends on a chain of events: escape; followed by spread; followed by harm.¹⁰⁸ Because risk is the function of two probabilities multiplied by each other (exposure x hazard), if the probability of either of these can be shown to be near zero, then the potential environmental risk is near zero. For example, if escape does not occur (i.e., representing the probability of exposure), there is no possibility for direct environmental harms to occur.

As discussed earlier in this section, several risk-related questions are important when considering the potential environmental risks of pigs with the IGA. These questions focus on the likelihood of escape and establishment (exposure) of pigs with the IGA in the affected environment, and, in the unlikely event that exposure occurs, the potential environmental consequences of this exposure. Information relevant to answering these questions has been presented throughout this EA and summarized in the sections above. This assessment has provided sufficient information to support that the likelihood of exposure (i.e., escape, survival, dispersal, reproduction, and establishment) of pigs with the IGA to the affected environment (Iowa and South Dakota) is extremely low; thus, the risk to the affected environment is minimal. In addition, in the unlikely event of escape, dispersal and/or reproduction and establishment, the pigs with the IGA would not pose an environmental risk that

differs from the baseline risk of pigs without the IGA escaping and becoming feral. This conclusion also applies to unintentional release (e.g., malicious activities and natural disasters).

5.3 Risk of the presence of the *nptII* gene and NPTII protein to the affected environment

As discussed previously, the construct that remains in GalSafe® pigs contains the *nptII* gene, which expresses the NPTII protein. Therefore, the gene and protein may be present in the manure, carcasses, or any other remnants of GalSafe® pigs. As discussed previously, pig carcasses would be disposed of by composting, incineration, or rendering. If pig carcasses or other animal remnants are disposed of by incineration, then the *nptII* gene and NPTII protein from GalSafe® pigs would be destroyed and would not enter the environment. On the other hand, the gene and protein may be present in the manure applied to soil, or carcasses or other remnants of GalSafe®, which are composted and disposed of in the affected environment surrounding the single production facility in Iowa and the single abattoir in South Dakota. In addition, if tissues of homozygous GalSafe® pigs are rendered, the *nptII* gene and NPTII protein may become incorporated into rendered products if they survive rendering processes. As previously stated, rendered products can be used in animal feed; however, rendered products from homozygous GalSafe® pigs would be expected to be dispersed and disposed of on a wider geographic scale. These potential environmental exposure pathways of manure application to land, composting animal carcasses or any other animal remnants, or disposal of rendered products are considered when evaluating the risk questions in the following discussion.

5.3.1 What is the risk from toxicity due to the presence of the *nptII* gene and NPTII protein to the affected environment?

The *nptII* gene consists of DNA. DNA is ubiquitous in the environment and DNA, when consumed in the diet, has no direct toxic effect (i.e., it is considered generally recognized as safe or GRAS). The presence of the *nptII* gene in the environment from the disposal of manure, carcasses, or other remnants of GalSafe® pigs will be minimal compared to the total DNA already present in the affected environment. Also, the DNA in the *nptII* gene has no unusual composition compared to other genes. Together, these factors effectively minimize the risk of toxicity from the presence of the *nptII* gene in the environment.

Proteins are also ubiquitous in the environment. In particular, the NPTII protein is widespread in the environment and in food chains, in the form of naturally occurring resistant microorganisms found in soil and mammalian digestive systems, and is not expected to result in toxicological risk to organisms.

Fuchs et al.⁶⁴ reported no acute toxicity to mice fed NPTII protein via gavage at exaggerated doses (as high as 5000 mg/kg body weight). Hily et al.⁶⁶ examined the soil microbiome surrounding genetically engineered (*nptII* gene with the coat protein gene of *Grapevine fanleaf virus*) and nonengineered grapevine root stock after 6 years and noted no differences in soil bacteria between the two groups – the presence and expression of the *nptII* gene did not disturb the composition of nontargeted bacterial communities. After reviewing the safety data on the NPTII protein in plant material, the EPA concluded that residues of the protein are exempt from the requirement of a tolerance in all food commodities when used as a plant-incorporated inert ingredient (40 CFR 174.521).¹¹⁷ Additionally, in general, proteins are known to degrade rapidly in the environment in part due to the ubiquitous presence of enzymes (e.g., proteases and peptidases) produced by microorganisms and fungi in the manure and environment.¹¹⁸⁻¹³¹ For example, the estimated bioactivity half-life of the Bt protein (*Bacillus thuringiensis* Subspecies *kurstaki* Cry1Ab or CryIIA insecticidal protein) from transgenic plant tissues cultivated into soil ranged from 1.6 to 31.7 days.^{132, 133} Therefore, the NPTII protein is expected to degrade relatively rapidly in the environment, limiting its environmental exposure.

Conclusions: The risk of toxicity due to the presence of the *nptII* gene and the NPTII protein to the affected environment is extremely low. DNA, such as the *nptII* gene, do not pose a significant toxicological risk to the affected environment. DNA is ubiquitous in the environment and dietary DNA has no direct toxic effect. The DNA in the *nptII* gene has no unusual composition compared to other genes; it is composed of four nucleotides common to all genes in all organisms in varying amounts. Its presence poses no more toxicity than any other DNA that is ingested.¹³⁴ As described above, the NPTII protein is also widespread in the environment, expected to degrade rapidly, and not expected to result in toxicity to organisms in the environment.

5.3.2 What is the likelihood of increased antimicrobial resistance in the affected environment occurring due to the possible presence of the *nptII* gene and the NPTII protein in manure and other waste products of GalSafe® pigs?

Antimicrobial resistant bacteria are naturally present in the environment. For example, several classes of antimicrobial resistance genes and their expression products, including some examples of *nptII*, can be found in environmental samples, including soils^{135, 136}, a coastal plain stream¹³⁷, river water¹³⁸, sewage¹³⁸, sewage sludge¹³⁹, and manure.^{138, 140-142} It has also been estimated that one out of every 10⁴-10⁶ bacteria in the microbiome of pigs in agricultural use contains an antibiotic resistance gene, with no differences in quantity between organic and conventional agricultural methods.¹⁴³ In

addition, 2.3 to 15.6% of cultivable soil bacteria which carried antimicrobial genes, including but not limited to the *nptII* gene, were naturally resistant to kanamycin or neomycin.¹⁴⁴ Therefore, the presence of the *nptII* gene and the NPTII protein in manure, carcasses, and other animal remnants of GalSafe® pigs will not introduce new genes or proteins into the environment. The question then is the likelihood of whether there will be an increase in antimicrobial resistance in the affected environment.

The NPTII protein is the expression product of the *nptII* gene and is what confers phenotypic resistance to some aminoglycosides in bacterial species containing the *nptII* gene. However, the presence of the NPTII protein alone in the affected environment (either produced by the tissues of GalSafe® pigs or produced by bacteria that may have acquired the *nptII* gene) is not expected to result in an increase in bacterial populations that possess antimicrobial resistance. Therefore, the discussion below will focus solely on the likelihood of the *nptII* gene to increase antimicrobial resistance in the affected environment.

The *nptII* gene in the *pPL657* rDNA construct is ubiquitous in the tissues of GalSafe® pigs. The presence of the *nptII* gene has the potential to contribute to the development of antimicrobial resistance through horizontal gene transfer in the gut of GalSafe® pigs, in manure from GalSafe® pigs, or in the environment if manure or other wastes (pig carcasses and any remnants) are deposited in the soil environment. Horizontal gene transfer is the movement of genetic material between unicellular and/or multicellular organisms other than by transmission of DNA from parent to offspring. Horizontal gene transfer can occur through three distinct mechanisms: 1) conjugation, 2) transformation, or 3) transduction. Conjugative transfer is a process that requires cell to cell contact and formation of a pore between the donor and recipient cell, through which DNA or mobile genetic elements (e.g., genes, cassettes, or chromosomal DNA) can transfer. Transformation results from the uptake of extracellular, free floating DNA by a bacterial cell. In a bacterial population, some cells will be capable of such uptake (i.e., will be competent) while others are not. Once this DNA becomes intracellular, integration into the bacterial genome relies on homologous recombination, which requires some regions of shared sequence between the foreign DNA and the bacterial host's genome, or less frequently can be accomplished with illegitimate recombination (non-homologous recombination). Transduction involves bacterial gene transfer via a viral vector (e.g., bacteriophage). A phage infecting a bacterium can incorporate bacterial DNA and pass it along as it infects other bacterial cells.

Manure, carcasses, or other animal remnants from GalSafe® pigs may contain the *nptII* gene¹⁴⁵ or bacteria that have acquired the *nptII* gene via horizontal transfer from pig cells in the gut of the pigs

with the IGA. If manure or composted carcasses/remnants of GalSafe® pigs are applied to the soil environment, the introduction of the antimicrobial resistant bacteria and the *nptII* gene into the soil could potentially contribute to an increase in the population of antimicrobial resistant bacteria in the local environment (i.e., the soil environment where manure, carcasses, or other animal remnants are applied). However, the likelihood of an increase in the population of antimicrobial resistant bacteria to occur would be limited for several reasons. First, the likelihood of the spread of antimicrobial resistance to additional bacteria by conjugation is much lower in the soil than would be expected in the gut of the pig.^{146, 147} Second, one of the primary mechanisms of horizontal gene transfer that could apply in the soil environment is natural transformation. In order for transformation to occur, several coexisting conditions have to be met: there must be DNA present in the extracellular environment; the recipient bacteria must be in a state of competence; and the translocated DNA must be stabilized, either by integration into the recipient genome, or by recircularization (in the case of plasmid DNA). Each condition must occur concurrently, therefore, the likelihood for this combination of conditions to occur at the same time for natural transformation is limited. Third, the likelihood of resistant bacteria entering the environment through the gut of GalSafe® pigs will be further minimized because the current lineage of GalSafe® pigs has not been exposed to aminoglycoside antibiotics in any of the generations since insertion of the IGA, thereby eliminating the possibility of selective pressure from aminoglycoside antibiotic challenge. There will also be a statement on the label of GalSafe® pigs that restricts the use of aminoglycosides in these animals. Although it is possible that manure from GalSafe® pigs could be mixed with manure from other animals containing aminoglycoside antibiotics, the restrictions for use of aminoglycoside antibiotics in GalSafe® pigs nevertheless helps limit this possibility.

Rendering is generally accomplished with steam at temperatures of 115°C and 145°C for 40 to 90 minutes, depending on the type of system and materials.⁷⁴ DNA generally denatures at temperatures between 60°C and 110°C.¹⁴⁸ Therefore, it is unlikely that the intact *nptII* gene will survive the rendering process. If the intact *nptII* gene survives the rendering process, it is also possible that the *nptII* gene could be present in rendered products of homozygous GalSafe® pigs. If the intact *nptII* gene is present in rendered products, then the gene could also be introduced to the environment via disposal; for example, the *nptII* gene could be contained in manure from animals fed rendered products derived from homozygous GalSafe® pigs. However, in addition to the factors described above that would limit the potential for horizontal gene transfer, the possibility of rendered products from homozygous GalSafe® pigs increasing populations of antimicrobial resistant bacteria are even more remote due to the

rendering processes (which may destroy or denature the *nptII* gene) and the expected disperse distribution and disposal of the rendered products.

Conclusions: As noted above, the presence of the NPTII protein is not expected to result in an increase in antimicrobial resistance in the affected environment. It may be possible for the presence of the *nptII* gene in GalSafe® pigs to lead to an increase in antimicrobial resistance in the local environment (i.e., the soil environment where manure, carcasses, or other animal remnants are applied). However, antimicrobial resistance occurs naturally in the environment and the likelihood of an increase of antimicrobial resistance occurring in the local environment is limited for the reasons stated above (e.g., conjugation would be lower in the soil than in the gut of the animal, the occurrence of natural transformation in the soil will be limited, and GalSafe® pigs will not be exposed to aminoglycoside antibiotics).

5.3.3 Conclusions on risk of increased antimicrobial resistance in the affected environment occurring due to the possible presence of the *nptII* gene and the NPTII protein in manure and other waste products of the GalSafe® pig

The presence of the NPTII protein alone is not expected to result in an increase in antimicrobial resistance in the affected environment. Conversely, the presence of the *nptII* gene could potentially lead to an increase in the number of microorganisms in the environment resistant to neomycin and other aminoglycoside antibiotics. However, even if this increase occurs, it is only expected to occur in the local environment (i.e., the soil environment where manure, carcasses, or other animal remnants from GalSafe® pigs are applied). In addition, the likelihood for an increase in antimicrobial resistance is limited because the r GalSafe® pig lineage has not been exposed to aminoglycosides, thereby eliminating the possibility of selective pressure from aminoglycoside antibiotic challenge, and restrictions preventing use of aminoglycoside use in GalSafe® pigs will be included on the drug label. Furthermore, GalSafe® pigs with the IGA will be housed at only one facility and slaughtered at only one abattoir, and the number of GalSafe® pigs in the herd will be limited to 1,000. Therefore, relatively few animal carcasses/remnants and relatively low amounts of manure would be generated and deposited into the soil environment. This will in turn result in limited potential exposure to antimicrobial resistant pathogenic bacteria in the local environment. Therefore, the risk is considered minimal for increased antimicrobial resistance in the wider affected environment from the presence of the *nptII* gene in manure, carcasses and other animal remnants of GalSafe® pigs.

5.4 Overall conclusion on the likelihood of significant impacts on the human environment

As stipulated by NEPA and its implementing regulation, major agency actions, such as new animal drug approvals, require that an assessment be made to determine whether the agency's action is likely to have a significant impact on the human environment of the United States. These are often derived from ecological risk assessments which "evaluate[s] the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors."¹⁴⁹ Risk is a function of hazard and exposure. In this EA, two general exposure pathways that could result in environmental impacts were identified:

1. The escape of GalSafe[®] pigs from a single production facility in Iowa and a single abattoir (slaughterhouse) in South Dakota into the affected environment, and
2. The introduction of the *nptII* gene and NPTII protein, resulting from the IGA, into the affected environment via manure or other wastes (including carcasses and any remnants) from GalSafe[®] pigs at the single production facility in Iowa and single abattoir in South Dakota.

However, due to redundant physical and procedural containment and limited exposure to wastes (manure, pig carcasses and any other animal remnants) produced at only two specific sites, which will be included in the conditions of approval, the exposure due to these two pathways will be limited. Thus, the likelihood of any associated risks is expected to be extremely low and significant impacts to the human environment would not be expected.

Section 6.0 Conclusion

The potential associated impacts from the production of GalSafe[®] pigs have been identified and evaluated herein. Due to redundant measures to prevent escape and the limited exposure to the *nptII* gene at only one production facility and one abattoir with a limited herd size (up to 1,0000 animals), the risks from these exposure pathways are expected to be minimal. Therefore, no significant environmental impacts are expected from the NADA approval of the *pPL657* rDNA construct in the genome of the GalSafe[®] pig line.

Section 7.0 Cumulative Effects

The proposed action would be the first NADA approval for the *pPL657* rDNA construct in the genome of the GalSafe[®] line of pigs and there are no concrete plans for herd expansion at the two

identified facilities or addition of new facilities at a specific location, therefore, no analysis of cumulative effects is needed.

Section 8.0 Alternatives to the Proposed Action

The only alternative to the proposed action is the “No Action” alternative which would be the failure to approve the NADA for the *pPL657* rDNA construct in the genome of the GalSafe® line of pigs. Under the “No Action” alternative, production of the GalSafe® line of pigs would likely be discontinued, and in that case, just as for the approval of the product, there would be no significant impacts on the quality of the human environment in the United States. Based on the analysis in this EA, significant environmental impacts are not expected to occur from the proposed action. Therefore, the “No Action” alternative was eliminated from consideration and further analysis.

Section 9.0 Agencies and persons consulted

This Environmental Assessment was prepared by Revivacor, Inc. with input on the scope and content from the Center for Veterinary Medicine at the Food and Drug Administration. No other Federal or state agencies or persons were consulted.

Section 10.0 List of Preparers

This document was prepared by employees of Revivacor, Inc.

Section 11.0 Certification

Revivacor, Inc. certifies that the information presented in this EA is true, accurate, and complete to the best of their knowledge.

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